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### 3' NONTRANSLATED RNA SEQUENCE AND STRUCTURE REQUIRED FOR REPLICATION OF HEPATITIS C VIRUS RNA IN CULTURED HUH7 CELLS.

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We carried out an extensive mutational analysis of the 3' nontranslated RNA (3'NTR) signals required for replication of subgenomic hepatitis C virus (HCV) RNAs in transiently transfected cells. The 3'NTR of HCV consists of a nonconserved region (ncr), a variable length poly(U/UC) track and a highly conserved, 3' terminal 98 nt segment (3'X). Previously, Yanagi et al. (PNAS 86: 2291-95, 1998) demonstrated that genome-length RNAs with large deletions in the poly(U/UC) region and 3'X were incapable of replication in the chimpanzee, while a 24 nt deletion in the ncr had little apparent effect on replication capacity. We confirmed these results and carried out a more detailed analysis of the requirements for structure and sequence within this segment of the HCV genome. **Methods:** To study the effect of 3'NTR mutations on RNA replication, we used subgenomic HCV-N replicons that encode the human immunodeficiency (HIV) virus tat protein and therefore induce expression of secreted alkaline phosphatase (SEAP) in proportion to the abundance of replicon RNA in transiently-transfected En5-3 cells (stably transformed Huh7 cells that express SEAP under control of the HIV LTR promoter). We analyzed sequence and structural requirements within the 3'NTR by introducing block mutations and single base substitutions using PCR and QuickChange mutagenesis. **Results:** RNA replication was undetectable in this system when any or all of the putative stem-loop structures within the 3'X region were deleted. Replication was also abrogated by block substitutions within either strand forming the duplex stem of the most 3' stem-loop (SL-1), and could not be restored by the creation of complementary block substitutions. The effects of individual base substitutions within the stem ranged from little influence on RNA amplification to the complete abrogation of replication. Among the single mutations that blocked replication, some compensatory mutations within the opposite strand that restored base-pairing also restored replication, while others did not. Thus the specific nucleotide sequence as well as the structure of the stem of SL-1 is critical for RNA replication. Single nucleotide substitutions within the loop of SL-1 adjacent to the stem also blocked replication, while substitutions in the apical three nucleotides were well tolerated. Most single nucleotide substitutions introduced into the putative SL-2 and SL-3 duplex stems resulted in the loss of replication, but none of these mutations could be rescued by compensatory mutations that restored base-pairing. A replacement analysis indicated that at least ~41 nts of the poly(U/UC) (from its 3' end) is required for replication. In contrast, multiple large deletion mutations were tolerated within the ncr region with only moderate loss of replication capacity (10% of wild-type). **Conclusion:** The 3' 140 nts of the HCV genome contain critical RNA sequences and structures that are essential for replication, while the upstream 3'NTR sequences contain accessory signals that support this function but can be deleted without completely abrogating replication.

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### EVIDENCE FOR A PROTEIN-MEDIATED 5'-3' INTERACTION OF THE BVDV RNA GENOME

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Following the entry into the host cell, the genome of (+)-strand RNA viruses exerts two major functions. Initially, it operates as a mRNA to yield the viral proteins. During replication, it serves as a template of the viral polymerase. The mechanisms controlling both competing processes are unknown but they are essential for the regulation of the virus life cycle. Experimental data obtained with the pestivirus BVDV (bovine viral diarrhea virus), a close relative of hepatitis C virus (HCV), suggest that both the termini of the viral genome participate in the translation as well as in the replication cycle. Thus, we could distinguish RNA motifs that are exclusively involved in either translation or replication. Moreover, we defined "bi-functional" signals in the 5' and 3' portion of the genome that modulate both processes. The latter signals are considered as important regulators between translation and replication - a scenario, which necessarily demands a functional cross talk between the viral RNA termini. We have obtained evidence to suggest that a 5'-3' communication of the BVDV genome is mediated by cellular proteins. Crosslinking and competition experiments with the 3' and 5' untranslated regions (UTRs) of the viral RNA revealed that a defined set of proteins specifically interact with elements of each of these regions. Interestingly, some of these elements correspond to the aforementioned "bi-functional" RNA signals. Mutations that impaired the protein interaction with the 3'UTR had severe consequences for the translation process and inhibited RNA replication. Most interestingly, enriched fractions of these proteins stimulated an association of the BVDV 5' and 3'UTR. These data strongly indicate the existence of a protein-bridge between the termini of the BVDV genome and suggest a closed loop formation as a prerequisite for the switch from translation to RNA replication.

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## 9th International Meeting on Hepatitis C Virus & Related Viruses

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### 3' TERMINAL BASE REQUIREMENT FOR HCV REPLICATION

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The 3'X region at the end of HCV genome is highly conserved including several 3' terminal bases and it is shown to play an essential role in HCV replication. We used the HCV subgenomic replicon system to systematically evaluate the role of the last 3' terminal bases (...AGU-3') in viral RNA replication. Over 30 specific changes were introduced by PCR into the 3' terminus of the replicon RNA including deletions, additions, substitutions, and double mutations. Many of the changes were designed to preserve the secondary structure of the 3' X region. Effects on replication of these modified replicon RNAs were assessed by a reporter-based assay as well as a colony formation assay. Our results showed that a pyrimidine base at the 3' terminal position (+1) is critical for replication. The change of the terminal U base to C was allowed and resulted in replication at comparable levels to the wild-type replicon, indicating that initiation of RNA synthesis by GTP is tolerated. However, sequence analysis of RNA isolated from the colonies produced by the replicon RNA revealed that the C base had reverted to the wild-type U base, indicating that despite efficient initiation at the C base, the terminal U base may provide a beneficial yet unknown advantage beyond the initiation stage. Any changes at the +2 (G) or +3 (A) position completely abolished replicon replication suggesting that these two bases are important for proper replication initiation. Consistent with our enzymatic characterization, strict purine bases at +2 and +3 positions are evolved to ensure initiation at the preferred terminal pyrimidine base. Similar base arrangement is also preserved at the 3' end of the (-)-strand RNA. Furthermore, deletions of the terminal U and GU bases were detrimental. Interestingly, insertion of a single pyrimidine nucleotide to the end of the replicon resulted in reduced but, nonetheless, significant replication. This suggests that internal initiation at a +2 pyrimidine base may occur, which would be detrimental and result in the loss of the terminal base if the genome ends with the authentic pyrimidine base. Increasing the number of bases added to the 3' terminus resulted in a proportional decrease in the replication efficiency. A model for terminal initiation will be presented in the context of both polymerase and viral RNA.

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### SUBGENOMIC HCV REPLICONS INDUCE A MEMBRANOUS WEB THAT IS INVOLVED IN RNA REPLICATION

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Plus-strand RNA viruses induce specific cytoplasmic membrane alterations that are indispensable for replication of the viral genome. For HCV, the intracellular site of RNA synthesis has not been defined and it is unknown whether modified cellular membranes are required for RNA replication. We have previously shown that expression of the entire HCV polyprotein in tetracycline-regulated cell lines induces distinct membrane alterations. A candidate viral replication complex was found to harbor all structural and nonstructural proteins and was designated 'membranous web'. The membranous web could be induced by NS4B alone and was very similar to the 'sponge-like inclusions' previously found by electron microscopy (EM) in the liver of HCV-infected chimpanzees. Here, we investigated whether intracellular membrane alterations occur during HCV RNA replication. **Methods:** Naive HuH-7 cells and HuH-7 cells harboring selectable subgenomic replicons (NS3-NS5B) were examined by indirect immunofluorescence microscopy (IF), EM, and immunocytochemistry EM (IEM). HCV nonstructural proteins were detected with monoclonal antibodies (mAbs) by IF in light microscopy (LM) and by immunogold labeling in the EM. HCV RNA was detected with a HCV-specific riboprobe of minus polarity either FITC-labeled for LM or digoxigenin- or biotin-labeled for EM in situ hybridization (ISH). HCV-specific RNA synthesis was located by BrUTP incorporation in the presence of actinomycin D. **Results:** By IF, HCV nonstructural proteins were found in a reticular staining pattern and in dot-like structures in the cytoplasm of HuH-7 replicon cells. At the ultrastructural level, these structures correspond to the membranous web previously identified in tetracycline-regulated cell lines. By IEM, the membranous web was found to contain all nonstructural proteins. Viral plus-strand RNA, detected by ISH, was also found on the membranous web. By double-IF, both newly synthesized viral RNA and proteins localized to similar dot-like structures. **Conclusion:** In cells harboring a subgenomic replicon the membranous web contains all nonstructural proteins, the bulk of plus-strand RNA, and newly synthesized viral RNA. Thus, the membranous web is the site of RNA synthesis and represents the HCV replication complex.

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